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CHEMICAL CONTROL OF THE EVISCERATION PROCESS IN *THYONE BRIAREUS* [1]

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Evisceration, in Holothuroidea, is a complex form of autotomy, involving expulsion of the viscera through a rupture in the body wall. It occurs in several species of sea cucumbers in response to a variety of noxious stimuli. Among more or less natural stimuli, fouled aquaria, high temperatures, and rough treatment are effective. Evisceration can be produced effectively in the laboratory by injection of strychnine or methylene blue (Pearse, 1909), injection of distilled water (Domantay, 1931; Dawbin, 1949), electrical stimulation, or immersion in a dilute solution of NH_4OH in sea water (Kille, 1931, 1935). In *Thyone briareus*, evisceration occurs through the anterior end and results in the loss of the aquapharyngeal bulb (or lantern) and its associated oral structures, as well as the viscera (Pearse, 1909; Scott, 1914; Kille, 1935). Generally, the lantern, the gut, and possibly the gonad are discarded together, neatly packaged in the weakened and stretched introvert portion of the body wall (Kille, 1935). For this loss to occur, the introvert must be ruptured, and the viscera and lantern freed from the internal support of mesenteries and retractor muscles.

The lantern is firmly attached to the body wall by the five pharyngeal retractor muscles. The anterior end of each muscle inserts on the anterior portion of one of the calcareous plates composing the lantern; the posterior end inserts at a distinct point on the superior surface of the longitudinal body wall muscle (LBWM). During evisceration, each lantern retractor muscle undergoes autotomy at its posterior terminus—the junction with the LBWM (Kille, 1935). Although the site of muscle breakage never changes, the timing of autotomy in the sequence of events comprising evisceration varies. All of the muscles may break even before swelling of the introvert begins, or they may break only after the process of evisceration is nearly complete (Kille, 1935). In one animal observed by Kille, two muscles were still attached after the initial swelling of the introvert, while the three other muscles were already autotomized. Thus, while the rupture of the introvert and autotomy of the PRMs are mandatory events in evisceration, they are partially independent.

In this report, the mechanical stresses involved in muscle autotomy are determined. Then, isolated pharyngeal retractor muscles are used to test for endogenous,

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autotomy inducing factors released by the evisceration stimulus. A preliminary report describing the assay system and the initial demonstration of such an endogenous factor was presented to the American Society of Zoologists (Smith, Greenberg, and Hill, 1971). We now describe the source and isolation of an endogenous factor capable of initiating the sequence of events leading to autotomy and evisceration.

Materials and Methods

Animals

Specimens of *Thyone briareus* were obtained from the supply department of the Marine Biological Laboratory at Woods Hole, Massachusetts. For our experiments at the Marine Biological Laboratory, the animals were maintained in trays of running sea water. In Toronto, specimens of *Thyone* were maintained in 25 gallon Instant Ocean culture systems at 16° C. Animals were always used within two weeks of delivery.

Medium sized specimens of *Mercenaria mercenaria,* also obtained from Woods Hole, were used for the clam heart bio-assay.

Anesthesia

Animals dissected without anesthetic always eviscerate. Even though the coelomic pressure necessary to complete evisceration is relieved by an incision through the body wall, the introvert portion of the wall loses tensile strength and becomes a soft gel (G. N. Smith, Jr., unpublished data). Moreover, the pharyngeal retractor muscles autotomize when such a cut is made. Fortunately, all the events of evisceration may be completely blocked by an appropriate anesthetic.

The anesthetic of choice is a 0.1% solution of chlorobutanol (1,1,1-trichloro-2-methyl-2-propanol; "Chloretone") in sea water. *Thyone* can be safely dissected after an immersion of only 7–8 min in this solution. Sea water, saturated with proplyene phenoxytol (Hill, 1966), is also effective. Treatment with this substance for 15 to 20 min produces adequate anesthesia, and animals recover after immersion for as long as an hour. Isotonic (0.5 M) $MgSO_4$ is an effective relaxant, but we no longer use it. This anesthetic is difficult to wash out. Furthermore, both the responsiveness of the muscles, and the condition of the connective tissue structures, may be modified by long exposure (30 min) to Mg^{++} ion.

In any case, exposure to the anesthetic should be as brief as possible to reduce the washout time and to ensure recovery of full muscle reactivity. Moreover, since effective exposure times are highly variable, the experimenter must follow the state of relaxation by palpating the animal with his fingertips. If an unrelaxed animal is gently pressed it will respond by contracting and stiffening to the extent that the calcareous lantern can no longer be felt through the body wall. A *Thyone* is relaxed enough for dissection when two successive attempts to feel the lantern through the body wall are successful.

Preparation of everted animals and of isolated pharyngeal retractor muscles

Thyone can be everted if it is first relaxed with a suitable anesthetic. The operation is performed by cutting off the cloacal end of the body, and pushing the lantern and viscera through the resulting opening with a finger or a glass rod.

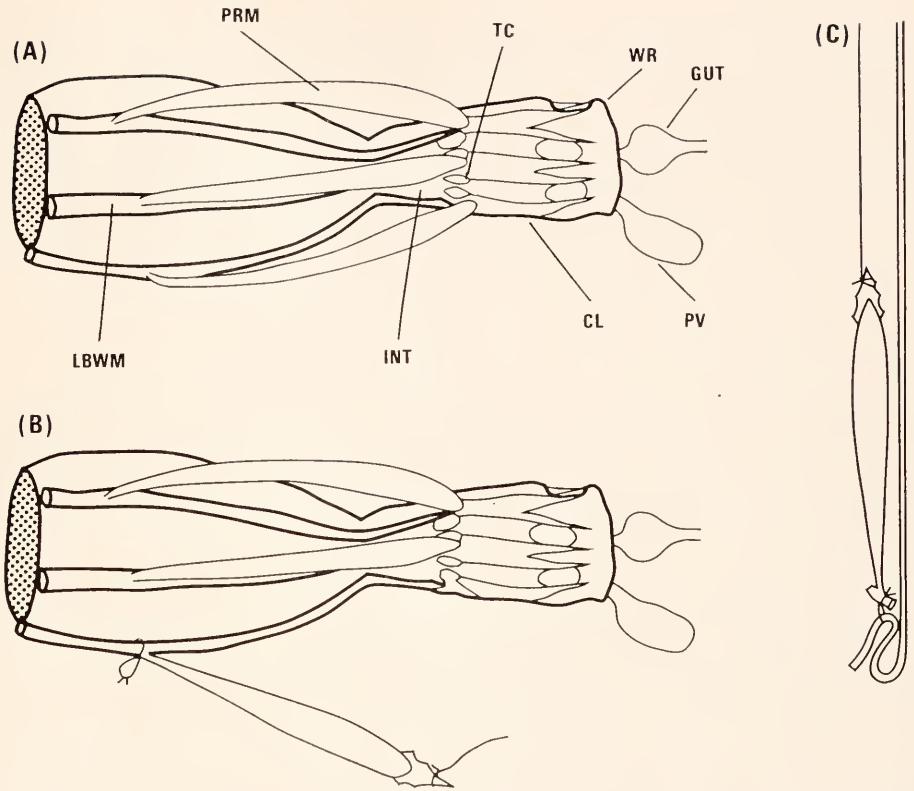

FIGURE 1. Preparation of isolated pharyngeal retractor muscles (PRM) from everted *Thyone;* (a) The relationship between the PRM and the longitudinal body wall muscle (LBWM). Note the calcareous lantern (CL), and the relationship between the tentacular canals (TC) and the anterior insertion of the PRM. Other abbreviations are PV, polian vesicle; WR, water ring; INT, introvert. (b) The anterior end of the PRM is freed by inserting a scissors in the tentacular canals and cutting a pie-shaped piece from the ossicle. Threads are tied near each attachment of the PRM: one on the ossicle and one on the LBWM; (c) The isolated PRM, tied to a stainless steel hook, and immersed in the testing bath (not shown). The upper thread is attached to a force-displacement transducer.

After the bulk of the viscera have been removed, the lantern, retractor muscles, and the body wall are washed with sea water. After sufficient washing, the animal will recover without undergoing either autotomy of the pharyngeal retractor muscles (PRMs) or softening of the introvert wall.

If the isolated PRMs are to be prepared, the freshly everted animal is washed with anesthetic solution instead of sea water. The lantern attachment of the PRM can then be freed by inserting the sharp point of a fine scissors in the exposed tentacular canal on either side of the anterior insertion of the muscle and cutting a wedge shaped piece from the calcareous plate of the lantern. Once the piece of lantern has been cut away from the introvert, the anterior end of the PRM is detached (Fig. 1b). A thread is attached to the lantern fragment.

Next, a fine forceps is forced under the longitudinal body wall muscle (LBWM) posterior to its junction with the PRM. A thread is pulled under the LBWM, and tied firmly around the muscle. A small loop is tied in this thread to facilitate attachment of the muscle to a hook in the bottom of a muscle bath (Fig. 1b). The junction of the PRM and the LBWM is freed by carefully cutting through the LBWM anterior to the junction, then cutting the LBWM away from the body wall with fine scissors, and finally cutting through the LBWM again, posterior to the thread. Now, the muscle is free, and both insertions are intact and undamaged. The entire preparation can be suspended between a stainless steel hook and a force-displacement transducer in an aerated, temperature-regulated organ bath. The bath fluid used was either natural sea water (at Woods Hole) or artificial sea water (Harvey, 1945).

Many PRMs are subdivided into 2–8 distinct bundles posterior to their anterior insertion on the lantern (see also Pople and Ewer, 1954; *Cucumaria sykion*). An animal with multiple bundles in one PRM usually has the same number of bundles in all of its five muscles. Although each bundle has its own insertion on the longitudinal body wall muscle, the individual junctions are tightly grouped. PRMs with multiple bundles were dissected and treated as single muscles.

Bioassay of acetylcholine

Isolated ventricles of *Mercenaria mercenaria* were used to assay acetylcholine in tissue extracts. The classic preparation of Welsh and Taub (1948) was employed. Benzoquinonium chloride (Mytolon) (Sterling-Winthrop) was used as a specific antagonist of the acetylcholine response.

Isolated PRMs were also used as ACh assay objects. The effect of ACh—a contracture—develops rapidly, is graded according to dose, and can be washed out quickly at any time during the response. Threshold is about 10^{-9} M, without eserine treatment, and the responses are repeatable over a long period of time.

Preparation of extracts

Hot distilled water was added to tissue in a glass homogenizer (1 ml per gram fresh tissue; 1 ml per 0.01 gram lyophilized tissue) and the mixture heated in a boiling water bath for three minutes. After vigorous homogenization with a motor driven Teflon pestle, the extract was heated for three minutes more.

The homogenized, boiled samples were cooled and centrifuged at 4° C, for 20 minutes at 27,000 × *g* (Sorval Model RC-2B). The supernatant fluid was retained. Those extracts not assayed immediately were frozen and stored at −20° C. Preparations stored 3 months were still active. Some extracts were lyophilized to concentrate the activity.

Gel filtration

Active extracts were fractionated by column chromatography (Sephadex G-15) with a 0.025 M phosphate buffer (pH 7.0) as the elution fluid. The separation was carried out in two steps. The initial fractionation was on a short column (2.6 cm × 30 cm). The resulting active fractions were combined, lyophilized, and rechromatographed on a long column (1.6 cm × 88 cm). All chromatography was carried out in a cold room at 4° C. The volume of sample applied was 1.5 ml on the short column, and 0.5 ml on the long column.

Five milliliter fractions were collected automatically and assayed on the PRM and the clam heart. The buffer affected neither assay object.

Recording

Responses of the clam heart and the PRM to contracture inducing agents were recorded on either a Grass polygraph (Grass Instrument) or a Physiograph Four (Narco BioSystems). The Grass recorder produces curvilinear recordings; the Physiograph, rectilinear recordings. Either system gave easily recognizable, unique response patterns for the agents assayed.

Results

The stimulus for evisceration and autotomy in normal and everted animals

Evisceration of intact *Thyone* could be induced by either of the two classical techniques of Kille (1935): immersion in ammonia water (1 ml of 7 N NH_4OH in 800 ml of sea water) or electrical stimulation. Electrical stimuli (50 V; 6 msec; 4/sec) were delivered for 30–60 sec to the external surface of the body with a square wave stimulator (Grass Model SD9) equipped with Ag-AgCl electrodes. Some variation in threshold was seen, but long duration and low frequency were important characteristics of successful stimuli. If, when electrical stimulation failed, it was followed immediately by gentle manipulation, evisceration rapidly ensued.

On the hypothesis that the mechanism of electrically induced evisceration is massive stimulation of the nervous system, KCl was introduced into the coelom as a possible evisceration stimulus. In fact, intraperitoneal injection of 0.5–2 ml of isotonic (0.54 M) KCl solution always produced rapid evisceration, followed by quick recovery and apparently normal regeneration of the treated animals.

If evisceration is caused by nervous stimulation, then anesthetics should block the response. Accordingly Mg^{++} ion, chlorobutanol and propylene phenoxytol were tested. All three substances were effective antagonists of evisceration either by electrical stimulation or by KCl injection.

Everted animals, stimulated either electrically or with KCl, demonstrate the evisceration response (G. N. Smith, Jr., unpublished data). Not only introvert softening, but also PRM autotomy, can be observed more clearly in such preparations. To test the specificity of KCl as an evisceration stimulus, solutions of common sea water ions, distilled water, and acetylcholine were tested. The ionic solutions, KCl, NaCl, $MgSO_4$, $CaCl_2$, NH_4Cl, and Ca^{++}, Mg^{++}— free sea water, were isosomotic with sea water. One tenth of the animal's volume was injected into intact *Thyone,* while 1/10 of the total volume was added to the bathing fluid containing everted preparations.

Of all the solutions tested, only KCl was effective as a stimulus of both intact and everted sea cucumbers. Ammonia water seldom elicits evisceration when applied intraperitoneally, and has no action when applied directly to everted preparations. Thus, ammonia applied to the external surface may act by stimulating cutaneous sensory structures. Distilled water, an occasionally effective eviscerant when injected, presumably acts by osmotically stimulating nervous elements.

Acetylcholine, whether injected or directly applied, causes strong contractions of the body wall musculature, but never evisceration. Doses as high as 10^{-4} M were tested.

The site of PRM autotomy in normal and everted animals

During evisceration, autotomy always occurs at the junction of the pharyngeal retractor muscle (PRM) with the longitudinal body wall muscle (LBWM). Careful examination of twelve animals eviscerated by immersion in ammonia water showed 60 clean breaks; all were at the junctions. During the course of this study, and others, hundreds of animals have been stimulated to evisceration by electrical shock, distilled water injection, rough treatment, polluted aquaria, immersion in dilute NH_4OH, and KCl injection. In no case did breakage ever occur at any site but the junction.

Since only the weight of the lantern opposes PRM shortening in everted preparations, the break at the junction must occur at very low tensions. However, in spite of these low break tensions, and although the junction appears to be a predetermined fracture plane, it is not a site of mechanical weakness. Three relaxed animals were everted, but the $MgSO_4$ solution was not washed out. The anterior end of the passive muscles, together with their attached ossicles, were freed and pulled by hand until they broke. The 15 muscles treated in this manner never broke at the junctional fracture plane. Thirteen tore near the mid-point of the muscle, and two failed at their insertions on the calcareous plate.

Autotomy in isolated retractor muscles

An isolated PRM, exposed to 100 mM KCl in the bathing solution, contracts and then autotomizes (Fig. 2). A similar response may be obtained by electrical stimulation. Autotomy can be blocked by anesthetising the muscle before stimulating it. The break always occurs at the junction of the PRM with the LBWM (*e.g.*, Table I).

Acetylcholine, in doses as high as 10^{-4} M in the bath, contracts the muscle but does not produce autotomy. Two such PRMs remained in ACh contracture for 6 hr 28 min, and 7 hr 43 min respectively; finally they tore, but in the middle of the muscle.

Therefore, insofar as they respond to the same stimuli, the autotomy of isolated PRMs is analogous to that in whole or everted *Thyone*.

Comparison of passive and autotomy breakage tensions

Breakage tension, during both passive stretch and autotomy, was also measured on unanesthetized, responsive, isolated retractor muscles.

Passive tension was developed by a spring loaded device which broke the muscle by stretching it 5 cm in 0.5 sec. Fifteen muscles (including those in Table I) were passively stretched. The mean breakage tension was 110.0 ± 17.8 grams ($\pm$ standard deviation). Tension was approximately proportional to muscle size. All but three of the breaks were at sites other than junctions.

Autotomy breakage tension was measured by allowing KCl treated muscles to contract isometrically until they failed. In each of four animals, one pair of muscles was treated with KCl, while two others were passively stretched. The resulting breakage tensions and sites of breakage, for each muscle, are displayed in Table I. The bundles comprising the PRM usually break in concert. In some instances, however, bundles broke separately (Table I, muscle 1E; Figs. 2 and 7).

TABLE I

Breakage tensions of pharyngeal retractor muscles during KCl-induced autotomy and under passive stretch

Animal	Muscle*	Weight of muscle (mg)	No. of bundles	Treatment	Breakage tension (g)	Breakage site
1	C	57	2	KCl	8	Junction
	A	46	2	KCl	3.5	Junction
	E	60	2	Stretch	90†	PRM
					35	Junction
	B	58	2	Stretch	100	PRM
2	B	15	1	KCl	<0.1	Junction
	A	25	1	KCl	<0.1	Junction
	E	24	1	Stretch	30	Junction
	C	29	1	Stretch	50	Ossicle
3	B	34	3	KCl	2.75	Junction
	D	53.4	3	KCl	5	Junction
	A	24.4	3	Stretch	180	Ossicle
	E	31	2	Stretch	300	PRM
4	B	56	3	KCl	4	Junction
	A	37.9	3	KCl	5.5	Junction
	D	62.1	3	Stretch	100	LBWM
	E	73.7	2	Stretch	120	Ossicle

* Muscle nomenclature after Carpenter, 1884 (in Hyman, 1955).
† Two bundles broke separately.

Ten muscles treated with KCl (including the pairs in Table I) developed tensions isometrically and broke in 6–10 sec. The mean breakage tension of autotomy was 3.9 ± 1.04 grams. However, muscles contracted by KCl actually lose tension before breakage occurs. The breakage shown in Figure 2, at high chart speed, would be recorded as having occurred at 5 g tension, although the muscle actually tore at about 1 g. Therefore, the measurements used to calculate the mean breaking tension (*e.g.*, Table I) tend to be high.

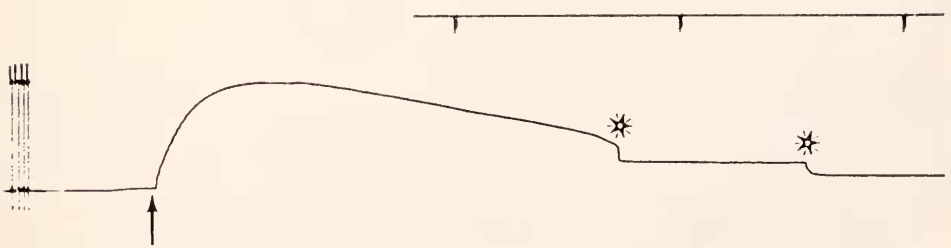

FIGURE 2. KCl induced autotomy of an isolated two-stranded pharyngeal retractor muscle of *Thyone*. KCl concentration of the bath increased to 100 mM, at the arrow. Stars indicate autotomy of the individuals muscle bundles; tension calibration: 5 g; time mark: 5 sec; rectilinear recording.

Muscles treated with KCl also break under isotonic conditions. PRMs loaded with 250–750 mg tear in 60–180 sec.

The evisceration factor

As the introvert of an eviscerating *Thyone* ruptures, quantities of coelomic fluids are expelled. A sufficiently large amount of this material, injected into a whole animal, can stimulate evisceration of the recipient. In contrast, the coelomic fluid of normal, intact *Thyone* never evokes the evisceration response. These results suggest that an endogenous evisceration factor (EF) is present in the coelomic fluid of eviscerating animals.

Coelomic fluid from eviscerating animals causes slow, rhythmical contractions in isolated PRMs; if the EF activity of the fluid is sufficiently high, autotomy occurs. The contraction produced by EF-containing fluid develops slowly, in contrast with ACh contractures (Fig. 3). Furthermore, once maximum tension has developed,

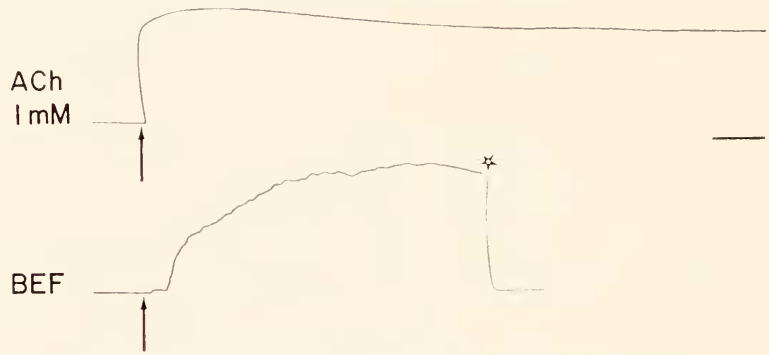

FIGURE 3. Responses of isolated pharyngeal retractor muscles of *Thyone;* (a) Effect of acetylcholine (ACh); (b) Effect of boiled evisceration factor (BEF). Substances were added at the arrows. Star indicates autotomy of the muscle, time mark: 10 sec; curvilinear recording.

the muscle relaxes along a time course similar to its contraction. The EF induced contraction also differs from that of ACh in its washout characteristics. ACh washes out much more rapidly than EF. Furthermore, if EF is washed during contraction, the contraction continues about to the level which would have been reached in the continued presence of EF. If the bath fluid is not changed, the muscle will enter successive cycles of contraction and relaxation, and will continue to show this slow, rhythmic activity until autotomy supervenes. The moment of autotomy for an isolated PRM depends on the EF activity administered. High concentrations of the factor produce breakage during the first contraction (Fig. 3). Less active preparations induce autotomy later in the series of contractions. If very low concentrations are given, autotomy may not occur at all. One preparation underwent 22 contractions over a period of 8 hr 9 min without breaking.

Autotomy need not occur at the maximum tension developed by the muscle. For example, since the waves of contraction diminish, with time, in both amplitude and duration, breakage may occur at a tension less than the maximum developed during the previous cycle. Furthermore, autotomy may occur on the relaxation as well as on the contraction phase of a cycle.

Autotomy inducing activity can be detected in the coelomic fluid extruded into the swollen introvert immediately on evisceration, but much higher activities develop if the eviscerated organs are allowed to steep in the fluid. Thirty minutes after evisceration, activity in the coelomic fluid is always high. If, however, active coelomic fluid, with the viscera removed, is allowed to stand at room temperature, the activity decays. This decay is not prevented by centrifugation at $27{,}000 \times g$ to remove coelomocytes, but is prevented by heating the coelomic fluid in a boiling water bath for a minimum of three minutes. Longer boiling (10 to 15 min) diminishes activity.

The relative heat stability of the activity in coelomic fluid suggests that the active substance is a small molecule. Dialysis of 25 ml of active coelomic fluid against 100 ml of distilled water at 4° C for 24 hours resulted in activity in both compartments. Further dialysis against 6 liters of distilled water completely eliminated the activity of the material in the dialysis bag. A sample kept in the cold room as a control and tested before and after the dialysis period retained its activity. Lyophilized material from the dialysate was tested in high concentration (*i.e.,* 50 ml of external dialysis medium were lyophilized and redissolved in 5 ml) and caused slow contraction and autotomy of isolated PRM.

These results suggest that evisceration activity is produced by one or more small molecules, and that these are inactivated by a soluble enzyme system.

Assay of the evisceration factor

The evisceration of whole *Thyone* is an ungraded, all-or-none phenomenon. Thus, many animals would be required to assay the activity of even one tissue extract. On the other hand, the effects of evisceration factor on PRMs are graded. Furthermore, one cucumber could provide up to 5 assay objects. Therefore, these muscles were conveniently used to assay the EF activity in the coelomic fluid of eviscerated animals and in tissue extracts.

Since autotomy of the muscle would terminate its usefulness, test solutions were sufficiently diluted so that they would not induce breakage on the first contraction. Furthermore, the muscles were washed out either before, or just at, the peak of the initial contraction. Thus, only the effectiveness of a particular extract as a contracture agent was measured.

In developing the PRM assay, we took advantage of two dose-dependent characteristics of the EF effect. First, the rate of contraction increases with activity; secondly, the delay between the administration of an active extract and the onset of contraction decreases as EF concentration increases. Some muscles may show only the rate or only the delay dose dependency (compare Figs. 4a and 4b).

The quantitative effect of the same dose of active material on the same muscle are not precisely repeatable. In view of this lack of precision, a set of responses of half-log-unit dilutions of haemal system extract was taken as the quantal responses of each assay muscle (Fig. 4). The effects of all other extracts on that muscle, were compared with these quantal responses. By means, the values shown in Table II were determined.

The source of the evisceration factor

Since activity develops slowly in evisceration fluid only if fragments of the viscera are present, extracts of ejected organs were tested for EF activity.

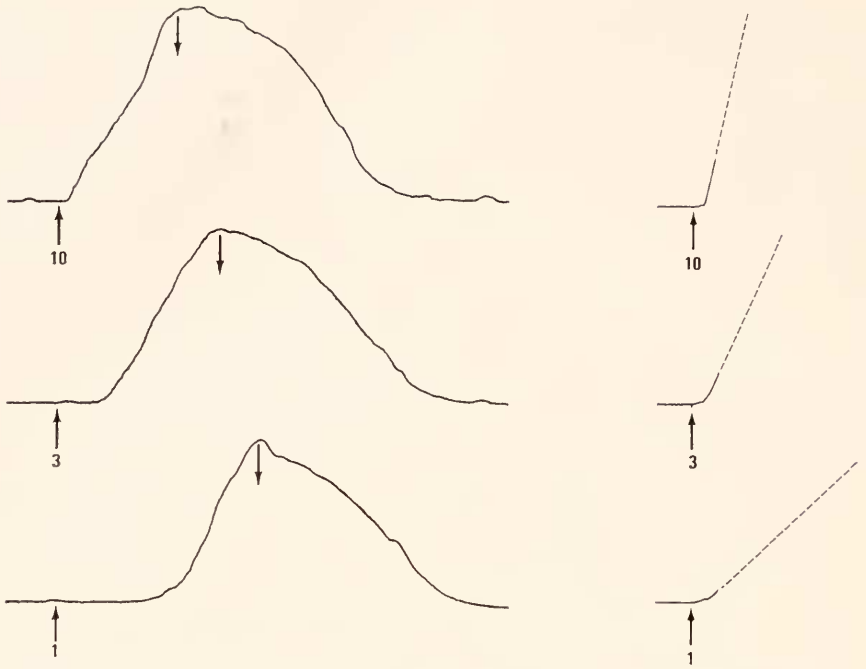

FIGURE 4. Standardization of evisceration factor bioassay on two isolated pharyngeal retractor muscles (PRM) of *Thyone*. Quantal responses are related to half-log-unit dilutions of haemal system extracts; quantal response of undiluted extract defined as 10. Doses (0.4 ml) administered to both PRMs: top records—undiluted haemal system extract (HS); middle records—3-fold dilution of HS (HS/3); bottom records—10-fold dilution of HS (HS/10). Note two types of responses: (A) delay between dose and contraction increases as dose decreases; (B) rate of tension development increases with dose. Extract added to the bath at upward-pointing arrows; washout at downward-pointing arrows; rectilinear recordings.

TABLE II

Relative activity of evisceration factor (EF), and concentration of acetylcholine (ACh), in tissue extracts from uneviscerated Thyone

Tissue	EF (relative activity*)	ACh (moles/liter)
Haemal system	10	10^{-12}–10^{-10}
Respiratory tree	1	10^{-9} –10^{-8}
Mesentery	1	10^{-9} –10^{-8}
Gut	0.3	10^{-9} –10^{-8}
Gonad	0.01	10^{-12}–10^{-10}
PRM	0.01	10^{-8} –10^{-7}

* Undiluted haemal system extract (10 mg/ml) is the standard preparation with a defined relative activity: 10. The relative activities of other undiluted extracts are given by the amount of haemal system extract necessary to match their effects. Thus, the activity of 10 mg respiratory tree equals that of 1 mg haemal system extract (see Fig. 4).

Ejected viscera were divided, immediately after evisceration, into four components: introvert, "lantern," "gut," and gonad. "Lantern" included both the PRM and the tentacles; "gut" included both mesentery and haemal system. These tissues were allowed to stand in 10 ml of sea water, at room temperature, for 30 minutes and then centrifuged. The supernatants were assayed but activity was detected only in the fluid in which gut was steeped. Extracts of the pellets were made in boiling sea water. Again, the "gut" extract was active, although low EF activity also appeared in the gonad pellet. ACh was detected in all tissues. Thus, of the ejected viscera, gut and its associated structures was the major source of extractable EF activity.

We next determined the distribution of the active component in intact organisms. Tissues from relaxed *Thyone* were prepared: extracts of gut, including mesentery and haemal system; isolated mesentery; isolated haemal system; respiratory trees; gonads; and pharyngeal retractor muscles were assayed on the PRM. Haemal system was the most active tissue (Table II) with respiratory tree, mesentery, and gut, showing mixed ACh and EF responses (Fig. 5). Haemal sys-

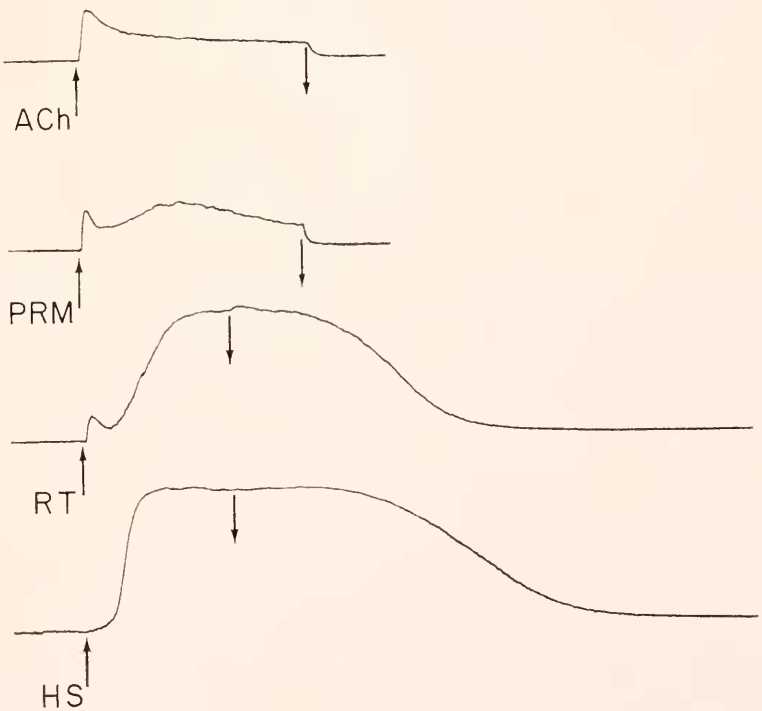

FIGURE 5. The effects of acetylcholine (ACh 10^{-7} M), and of evisceration factor (EF) from various tissues, on an isolated pharyngeal retractor muscle of *Thyone*. Extracts: pharngeal retractor muscle (PRM)—10 mg lyophilized tissue in 1.0 ml distilled water; respiratory tree (RT)—10 mg/ml; haemal system (HS)—1 mg/ml; doses of extract: 0.6 ml in 10 ml bath. Note that responses to PRM and RT contain both ACh and EF effects. Substances added at upward pointing arrows; washout at downward pointing arrows; time from beginning of record to dose: 100 sec; rectilinear recording.

tem sometimes has low ACh activity, detectable on the clam heart, but not on the PRM. The PRM extracts have low EF activity barely detectable in the presence of the high levels of ACh present.

Fractionation

Active evisceration fluid, and extracts of haemal system, respiratory tree, gut, and PRM were chromatographed on Sephadex G-15, with 0.025 M phosphate buffer as the eluent fluid. Only haemal system extract was placed on the long column directly. All other materials were first chromatographed on a short column; the active fractions containing both ACh and EF were lyophilized and resuspended in about 1 ml of water, then applied to the long column. All of the preparations contained an active factor which was identified as EF by its response on the PRM. The material eluted at a relative elution volume (Ve/Vo; where Ve = elution volume, Vo = void volume) of 1.58. The K_{av} was 0.36. (K_{av} is a partition coefficient defined as Ve-Vo/Vt-Vo, where Vt = total volume of the column.) The ACh eluted at a relative elution volume of 1.39 ($K_{av} = 0.225$). The peaks were close, but clearly separated (Fig. 6). ACh was identified by its characteristic effects both on isolated clam hearts and PRMs. Although 8 void volumes were tested, no other peaks active on the pharyngeal retractor muscle were found.

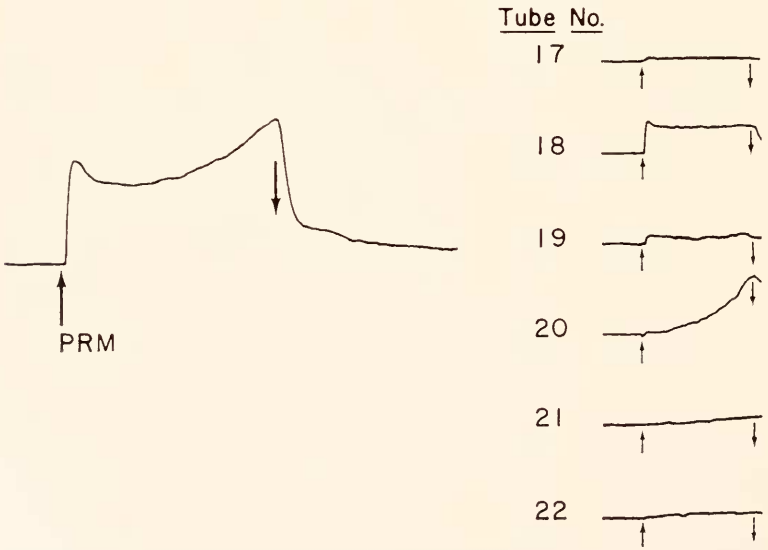

FIGURE 6. Fractionation of an extract of pharyngeal retractor muscle (PRM). Initial extract of lyophilized PRM in water passed through short column. Combined active fractions assayed (left-hand record); dose: 0.4 ml. Note both acetylcholine (ACh) and evisceration factor (EF) effects in the response. Combined active fractions passed through long column and assayed (right-hand records); dose: 1 ml of 5 ml fractions from long column. Tube 18 contains most of ACh; tube 20 contains EF. All assays performed on the same isolated PRM; tension amplification unvarying. Doses added at upward pointing arrows; washout at downward pointing arrows; rectilinear recording.

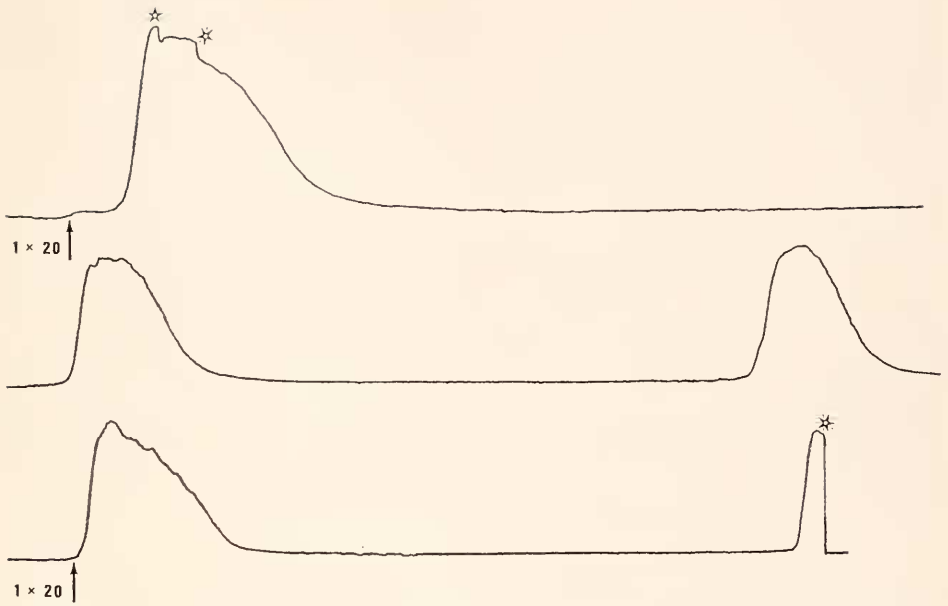

FIGURE 7. Autotomy of a three-stranded pharyngeal retractor muscle (PRM) induced by purified evisceration factor (EF). EF obtained from tube 20 of long column fractionation of haemal system extract (see Fig. 6); doses: 1 ml of the 5 ml fraction. Stars indicate autotomy of the individual muscle bundles; the third autotomy occurs only after a second dose of EF; tension at 1st peak is 5 g and tension at 3rd peak is 3 g; continuous record; total elapsed time: 2 hr, 18 min; rectilinear recording.

All tissue extracts producing the slow contraction waves in PRMs characteristic of EF yielded, on fractionation, the same component with activity identical to the whole extract. Large doses of this purified component induce autotomy (Fig. 7) and, after concentration by lyophilization, cause evisceration on injection into intact organisms. Probably, therefore, the EF activity of evisceration fluid is produced by a single molecule.

DISCUSSION

A factor inducing both autotomy of isolated pharyngeal retractor muscles and evisceration of whole animals appears in the coelomic fluid of *Thyone* upon evisceration. We have isolated this substance from the coelomic fluid as well as from tissue extracts. We propose that the many diverse conditions and compounds inducing evisceration all act through the common agency of this factor. Presumably the various stimuli release evisceration factor (EF) from intracellular stores, and the sequence of events comprising evisceration then follows perforce.

The factor would appear to be localized in nervous tissue since both KCl and electrical stimulation induce evisceration, while anesthetics block the effect. Although neither radial nerve cord nor the nerve ring is a very rich source of EF, additional nerve fibers are widely distributed in the organism. In particular, nerves have been demonstrated in the pharyngeal retractor muscles of *Thyone* by Margaret Hill in our laboratory at Woods Hole, and have been reported as well in

Thyonella gemmata (Nace, 1971) and in *Cucumaria sykion* (Pople and Ewer, 1954). Since, in addition, EF has been demonstrated in PRM extracts, the autotomy of isolated retractors following KCl or electrical stimulation is explainable on the mechanism proposed above.

Notwithstanding the evidence favoring EF localization in nerve, haemal system extracts were the richest source of the factor. Electron microscopy of the haemal system in *Cucumaria frondosa* revealed numerous bundles of axons in the vessel walls (Doyle, 1967). These neurons appeared to terminate adjacent to muscle cells, sometimes in deep invaginations in the fibers; but no membrane specializations were observed at the terminals. Large, membrane-bound, dense-core vesicles of unknown content occur in the nerve endings and Doyle (1967) suggested that these might be neurosecretory products. Assuming that the haemal system of *Thyone* is similar to that of *Cucumaria,* the high activity of EF in this tissue could be due to the high density of innervation or to the release of a specific neurosecretory substance. In any case, extracts of *Cucumaria frondosa* haemal system have yielded high levels of EF activity in preliminary experiments. The extracts were tested on *Thyone* PRMs and gave a typical EF response. The activity was recovered from the gel column at the expected elution volume.

Only the one substance, EF, is required to initiate the entire evisceration process including, not only autotomy of the pharyngeal retractor muscles, but also softening of the introvert wall. Exactly what the mechanism of EF action might be remains unclear. One hypothesis explaining the loss of tensile strength in an apparently collagenous structure would be the activation of a collagenolytic enzyme by the factor. However, no evidence that such an enzyme occurs in the introvert wall has been found (Smith, unpublished data). The wide tissue distribution of the evisceration factor, and its occurrence is a genus (*Cucumaria*) not normally exhibiting the evisceration response, suggests a further, more general role for the factor. In fact, evisceration may be only an incidental, if not accidential, effect of EF.

Low concentrations of EF induce cycles of slow contractions. Similar contractions have been described for the PRM of *Cucumaria sykion* (Pople and Ewer, 1958). These contractions are under nervous control, and the *Thyone* factor might be the neuromusclar transmitter by which they are effected. The effect of EF on other muscle preparations from *Thyone* is under investigation. The decay of activity at room temperature, and the preservation of activity by short boiling, suggests an enzyme system for removal of such a transmitter.

The electrical events associated with slow contractions are not known, but two types of membrane potentials have been observed in *Thyone* PRM in response to electrical stimulus (Prosser, Curtis, and Travis, 1951). A spike, apparently associated with twitch responses, can be measured. This response fades with increasing frequency of stimulation. In addition, a slow wave of potential may be recorded. Prosser *et al.* (1951) made no mechanical records corresponding to the electrical events, due to excessive fragility of the preparation. But, isolated PRMs from *Thyone* give vigorous contractions (>5 grams tension) on stimulation with ACh. The observed *in situ* fragility was probably due to the autotomy reaction, elicited by the electrical stimulus. In the same study, similar potentials were mea-

sured in *Phascolosoma* (=*Golfingia*) *gouldii,* a sipunculid. In this organism, slow waves were correlated with slow contractions.

The identity of the factor is unknown, but a number of relevant data are available. Neither ACh, 5HT, epinephrine, dopamine, L-dopa, norepinephrine, glutamic acid, 1-methyl adenine, nor histamine produce similar contractions or evisceration. No appreciable ninhydrin positive material occurs in the active fractions, and the absorption spectrum in the ultraviolet reveals no peaks. In particular, at 260 and 280 nm, the absorption is quite low. The material has either no effect, or a slightly excitatory effect (at high concentrations), on the clam heart. It elutes off the Sephadex column after ACh (MW = 181.6) but before leucine (MW = 131).

Evisceration in *Thyone* has been demonstrated in the course in Invertebrate Zoology at the Marine Biological Laboratory at Woods Hole for many years. Kille (1931; 1935) became interested in the problem during his association, as a student, with the laboratory. Our own work, although begun in December, 1970, was given impetus by our association with the Experimental Invertebrate Zoology course during the summer of 1971.

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Summary

1. The pharyngeal retractor muscles of *Thyone briareus* autotomize during evisceration at the junction of the PRM and LBWM. Autotomy involves a loss of tensile strength at the connection between the PRM and the LBWM.

2. An evisceration factor (EF) is present in the coelomic fluid expelled by eviscerating *Thyone*. This factor induces autotomy of isolated PRMs.

3. Most tissues have some EF activity, but haemal system is the richest source of the factor.

4. Purified EF causes evisceration on injection into intact animals.

5. The factor is a small molecule (~150 MW), of unknown character. It probably has other roles in muscle and connective tissue physiology.

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